In vitro study on the osteogenesis enhancement effect of BMP-2 incorporated biomimetic apatite coating on titanium surfaces

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To fabricate a sustained-release delivery system of bone morphogenetic protein (BMP-2) on titanium surface, explore the effect of BMP-2 concentration on the loading/release behavior of BMP-2 and evaluate the cell compatibility of the system in vitro, pure titanium specimens were immersed into supersaturated calcium phosphate solutions (SCP) containing 4 different concentrations of BMP-2: 0, 50, 100, 200 and 400 ng/mL. Biomimetic calcium phosphate coating was formed on titanium surface and BMP-2 was incorporated into the coating through co-deposition. The release profile of BMP-2 suggested that BMP-2 were delivered sustainably up to 20 days. CCK-8 and ALP assay showed that 200 group and 400 ng/mL BMP-2 group have significant effect on promoting MC3T3-E1 cell proliferation and differentiation. The BMP-2 incorporated into the hybrid coating released in a sustained manner and significantly promoted the proliferation and differentiation of MC3T3-E1 on the titanium surface.

Keywords: Titanium, Calcium phosphate coating, Bone morphogenetic protein 2, Sustained-release, Cell compatibility

INTRODUCTION

Titanium and its implants are widely used in orthopedic and dental field due to their excellent mechanical properties and biocompatibility. However, titanium itself is a bio-inert material and lack of bio-inductivity. To achieve successful osseointegration with surrounding tissues, strategies need to be applied to modify or improve the biological properties of the titanium surface to facilitate the growth and differentiation of osteoblasts and the subsequent tissue rehabilitation.

One of the possible effective therapeutics to functionalize titanium implant is to incorporate growth factors into the surface and create an active implant-tissue interface with the function of stimulating bone cell growth. Bone morphogenetic proteins (BMPs) are representative TGF-β superfamilly growth factors which affect cell growth, migration and differentiation. They also act as potent regulators during embryogenesis and bone/cartilage formation and repair[1-4]. Among them, BMP-2 has been approved by FDA to be used in the clinical treatment of spine fusion, tibia fractures and dental grafts[5,6]. By delivering BMP-2 to the local cellular environment, the bone repair process can be enhanced dramatically[7]. However, BMP-2 has a short biological half-time, is easy to be diluted or enzymolyzed by body fluid, and the efficacy of osteogenesis is highly influenced by its concentration and delivery mode[8]. It has been well understood that BMP-2 simply absorbed on the material surface releases very quickly and demonstrates a significant burst release phenomena, most part of the absorbed BMP-2 will be released during the burst release phase, which will result in the uneven distribution of protein concentration in different time period[9,10]. On the other hand, several researches have been carried out regarding to the dose effect of BMP-2 on the osteogenic differentiation and bone regeneration process[9-11]. An animal study using a rat calvarial defect model indicated that BMP-2 enhanced local bone reproduction in an osteoinductive dose threshold of 1.5–2.5 μg, no further bone formation enhancement or maturation was observed above this dose threshold; on the contrary, swelling, excessive bone formation or seroma formation emerged[9]. Meanwhile, it has been shown that high concentration of BMP-2 (500–2,000 ng/mL) tended to decrease periosteal cell proliferation and induce apoptosis in vitro, but, such reduction was not observed for both periosteal cell and preosteoblast at the low concentrations of BMP-2 (50–500 ng/mL)[10-12]. These results indicate that excessive BMP-2 compromises the osteogenic differentiation and bone regeneration process. For dental/orthopedic implant surgery, formation of the osteointegration between the titanium implant and the surrounding tissues takes about several weeks. Therefore, it is necessary to establish a proper delivery system by incorporating BMP-2 into a certain carrier matrix, through which BMP-2 can be slowly released at the bone/implant interface for a long period of time and in an effective and safe dosage, hence, making its osteogenesis function maximized.

Many organic and inorganic materials can be used to delivery BMP-2, including fibrous scaffold, silk fibroin scaffold, heparin conjugated poly(lactic-co-glycolic acid) (PLGA) nanospheres, collagen gels and so on. Both in vitro and in vivo experiments have demonstrated that good osteogenesis enhancement could be obtained by...
relatively low dose BMP-2 at nanogram level with the aid of the above delivery vehicles\textsuperscript{5,7,13,14}. Biomimetic calcium phosphate (CaP) coating is another type of effective carrier of proteins. It is produced by a solution-based method at the physiological condition, \textit{i.e.}, 37°C and pH 7.2–7.4. When titanium is immersed in a simulated body fluid (SBF) at the physiological pH value and temperature, a CaP layer can be formed on its surface\textsuperscript{15,16}. This CaP coating has been proved to be able to facilitate new bone formation and integration with surrounding bone tissue due to its chemical composition, biocompatibility and osteoconductivity\textsuperscript{17-20}. In addition, it is an ideal carrier for organic molecules that contain carboxylic groups such as BMP-2, vascular endothelial growth factor (VEGF) and antibiotics\textsuperscript{17,19,21,22}. When these organic molecules are dissolved in the saturated SBF solution containing calcium and phosphate, they can co-precipitate with the minerals simultaneously, since carboxylic groups have strong binding affinity to calcium ions\textsuperscript{18}. Previous study has demonstrated that recombinant human bone morphogenetic proteins 2 (rhBMP-2) incorporated in CaP coating is potent in stimulating the alkaline phosphatase (ALP) activity of the adhering cells\textsuperscript{23}. However, further information about the BMP-2 dosage, release profile and corresponding impact to the bone cells is still unclear. In this study, we aimed to fabricate a CaP-coated bioactive Ti surface with sustained BMP-2 release at a low dose level. BMP-2 was incorporated into CaP coating through biomimetic mineralization in SBF containing various concentrations of BMP-2. Surface properties of the CaP coatings, BMP-2 release profiles, and the effect of HA/BMP-2 hybrid coatings on osteoblast proliferation and differentiation were examined.

MATERIALS AND METHODS

Fabrication of BMP-2 loaded biomimetic HA coatings
10×10×0.1 mm\textsuperscript{3} titanium plates were machine-cut from medical pure titanium sheet (Baoji Qichen New Material Technology, Baoji, China). The Ti specimens were cleaned in acetone, ethanol, and deionized water successively for 20 min with a sonic oscillator. They were then immersed in 5.0 M NaOH solution and incubated at 80°C overnight. After the alkali etching treatment, the Ti plates were thoroughly rinsed with deionized water and air-dried at ambient temperature.

Biomimetic CaP coatings were fabricated on the etched Ti specimens through a two-way process reported before\textsuperscript{15}. In the first step, the Ti plates were washed with 75% ethanol for three times and sterilized under UV lamp for 10 min. They were then immersed into SBF at 37°C for 4 days to form a thin layer of amorphous CaP, which served as a seeding substrate for the growth of the crystalline layer containing BMP-2. In the second step, four concentrations of BMP-2, \textit{i.e.}, 0, 50, 100, 200 and 400 ng/mL, were added into supersaturated calcium phosphate solution (SCP) respectively. The Ti plates after the first step processing were immersed vertically into 4 mL the above SCP solutions with 4 BMP-2 concentrations, under vibrating (60 rpm) for 48 h at 37°C to form crystalline. After the two-step processing, the coated plates were then rinsed with deionized water and dried overnight at ambient temperature. All procedures were performed under sterile conditions. SBF and SCP were prepared based on the procedures described by Oyane \textit{et al.}\textsuperscript{24} (see Table 1). The pH value of SBF and SCP was adjusted to 7.4 at 37°C with tris-hydroxymethyl aminomethane and 1 M hydrochloric acid.

Characterization of biomimetic CaP coatings
The coated Ti plates were sputtered with gold particles. Surface morphology of the Ti plates was examined by scanning electron microscopy (SEM, Zeiss EVO18, Carl Zeiss, Oberkochen, Germany) at an accelerating voltage of 5 kV. The crystalline phases of the CaP coatings were determined by an X-ray diffractometer (XRD, Bruker D8, Bruker AXS, Karlsruhe, Germany). XRD was calibrated to cover the angular range from 20 to 60° in 0.04°. Chemical structure of the Ti surfaces after biomimetic mineralization was analyzed by Fourier-transform infrared spectroscopy (FTIR, spectrum 1000, PerkinElmer, Beaconsfield, UK). The CaP coatings on top of the Ti plates were gently peeled off from the Ti plates with a blade and grounded into powder. Transparent pellets were made by compressing a mixture of 150 mg KBr and 1 mg the above grounded powder. Each FTIR spectrum was a result of signal-averaging of 32 scans at a resolution of 4 cm\textsuperscript{-1} and the wave number range from 400 to 4,000 cm\textsuperscript{-1}.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Na\textsuperscript{+}</th>
<th>K\textsuperscript{+}</th>
<th>Ca\textsuperscript{2+}</th>
<th>Mg\textsuperscript{2+}</th>
<th>HPO\textsubscript{4}\textsuperscript{2−}</th>
<th>HCO\textsubscript{3}−</th>
<th>SO\textsubscript{4}2−</th>
<th>Cl\textsuperscript{−}</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBP</td>
<td>142.0</td>
<td>5.0</td>
<td>2.5</td>
<td>1.5</td>
<td>1.0</td>
<td>27.0</td>
<td>0.5</td>
<td>103.0</td>
</tr>
<tr>
<td>SBF</td>
<td>142.0</td>
<td>5.0</td>
<td>2.5</td>
<td>1.5</td>
<td>1.0</td>
<td>4.2</td>
<td>0.5</td>
<td>147.8</td>
</tr>
<tr>
<td>SCP</td>
<td>140.0</td>
<td>0.0</td>
<td>4.0</td>
<td>0.0</td>
<td>2.0</td>
<td>0.0</td>
<td>0.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>
The CaP coating was peeled off from the Ti plate and dissolved into 1 mL of 1 M acetic acid at 4°C for 30 min. One hundred microliter aliquots of each extract were transferred to 96-well microplates. One hundred microliter of anti-BMP-2 (Cloud-clone, Houston, TX, USA) were added to each well and the absorbance of the reaction product was measured at the wavelength of 450 nm using a spectrophotometer (Infinite 200, Tecan, männdorf, Switzerland). Readings were converted into BMP-2 concentrations by a calibration curve. The BMP-2 incorporation efficiency (IE) was calculated as follows:

\[
\text{Incorporation efficiency (IE)} = \frac{\text{Practical loading}}{\text{Theoretical loading}} \times 100\% 
\]

**In vitro BMP-2 release test**

The release profile of BMP-2 from the CaP coatings was determined by immersing the BMP-2 loaded Ti plate into plastic vials containing 2 mL phosphate buffer saline (PBS, pH 7.4) as a releasing medium. The sealed vials were then placed in a shaking water bath at 37°C. At the predetermined time points, the whole liquid in the vials was collected and refilled with 2 mL fresh PBS. One hundred microliter aliquots of the extracted liquid from each time point were assayed according to the aforementioned method in 2.3. Cumulative BMP-2 releasing amount was calculated and the cumulative release profiles in 20 days were plotted. The BMP-2 cumulative release rate was calculated as follow:

\[
\text{Cumulative release rate} = \frac{\text{Cumulative release amounts}}{\text{Practical loading}} \times 100\% 
\]

**Cell culture**

Mouse calvarial preosteoblastic cells (MC3T3-E1, Shanghai Chinese Academy of Science, Shanghai, China) were cultured in α-Minimal Essential Media supplemented with glutamax (Gibco, Grand Island, NY, USA) and 10% fetal bovine serum (FBS, Gibco) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The cell culture medium was changed every 3 days. When the cells reached 80–90% of confluency, a trypsin-glycerophosphate in order to induce osteogenic differentiation. MC3T3-E1 cells were seeded on the Ti surfaces at a density of 1×10⁵/mL. After 3 days, cells were fed with osteogenic differentiation media containing 50 μg/mL L-ascorbic acid and 2 mM β-glycerophosphate in order to induce osteogenic lineage. At day 7 and 14, the media and cells were collected for ALP activity assessment using an ALP kit (Jiancheng, Nanjing, China) according to the manufacturer’s instruction. Absorbance at 520 nm was measured with a spectrophotometer (Infinite200, Tecan). Total protein synthesis in the cell lysates was determined using BCA protein assay kit (Boshide, Wuhan, China). ALP activity was normalized to the total protein amount.

**Statistical analysis**

Statistical analysis was performed by SPSS software (SPSS v.17, IBM, Chicago, IL, USA). One-way analysis of variance was made for the data with equal variance (homogeneous variance), and Kruskal-Wallis test was used for the data with unequal variance (α=0.05).

**RESULTS**

**Morphology of biomimetic CaP coatings**

The morphologies of the biomimetic CaP coatings with various concentrations of BMP-2 observed under SEM were shown in Fig. 1. All the coatings displayed a rose-like appearance composed of nano-scale plates. No morphological difference was observed among the coatings with various BMP-2 concentrations and without BMP-2.

**Crystalline phase and chemical composition**

The X-ray diffraction profile of the deposited CaP coatings was shown in Fig. 2. Typical peaks of hydroxyapatite at 25.9, 28.2, 32.2, 34.1 and 49.5° in 2θ were found in XRD spectra, indicating that the coating was mainly consisted of hydroxyapatite. However, the amount of incorporated BMP-2 in CaP coating is not high enough to cause crystal phase change that could be detected by XRD.
Fig. 1 Scanning electron microscopy micrographs of coatings prepared in SCP containing different concentration of BMP-2. A. 0 ng/mL, B. 50 ng/mL, C. 100 ng/mL, D. 200 ng/mL, E. 400 ng/mL.

Fig. 2 X-ray diffraction profiles of coatings prepared in SCP containing different concentration of BMP-2. O: octacalcium phosphate (OCP), H: hydroxyapatite (HA), Ti: titanium. A. 0 ng/mL, B. 50 ng/mL, C. 100 ng/mL, D. 200 ng/mL, E. 400 ng/mL.

Fig. 3 FTIR spectra of coatings prepared in SCP containing different concentration of BMP-2. A. 0 ng/mL, B. 50 ng/mL, C. 100 ng/mL, D. 200 ng/mL, E. 400 ng/mL. (The bands at 1,032, 961.6 and 561 cm\(^{-1}\) correspond to P-O, 910 and 1,111 cm\(^{-1}\) bands are attributed to P-OH, and 872 and 1,418 cm\(^{-1}\) bands represent the existence of C=O.)

Therefore, no difference was found among the coatings with and without BMP-2. Chemical composition of the coating was verified by FTIR spectrum (Fig. 3). The bands at 1,032, 964 and 561 cm\(^{-1}\) correspond to PO\(_4^{3-}\). The 1,111 cm\(^{-1}\) is attributed to HPO\(_4^{2-}\) and 861 cm\(^{-1}\) indicates the existence of CO\(_3^{2-}\). Broad bands at 3,411 and 1,647 cm\(^{-1}\) are assigned to OH and amide I from BMP-2. These results further prove that the coatings are mainly consisted of carbonated CaP and the BMP-2 has been successfully incorporated into the coatings. The increase of the BMP-2 concentrations made no significant differences among these spectra.

In-vitro BMP-2 incorporation capacity and release profile

The IE of BMP-2 in the apatite coating was shown in Fig. 4. The value of IE demonstrated a positive correlation with the increase of BMP-2 concentration in the SCP solution. At the concentration of 50, 100, 200 and 400 ng/mL, the IEs were 8.0±1.8, 10.0±3.0, 11.0±1.7 and 11.7±0.8% respectively. The practical amount of BMP-2 in apatite coatings were 16.0±1.8, 40.1±3.0, 88.0±1.6 and 186.9±0.8 ng, respectively.

Figure 5 reveals the release profile of BMP-2 in PBS buffer from the hydroxyapatite layer. In all groups of BMP-2 incorporated coatings, the release of BMP-2 can be classified into two distinct stages. The first stage was a burst release stage which occurred in the first 3 days, with 29 to 51% BMP-2 being released out. The release amount and release rate of BMP-2 for the four groups were 8.3±0.1, 18.4±0.1, 33.7±0.5, 54.1±1.3 ng and 51.5±1.4, 46.0±4.0, 38.3±5.2, 28.9±1.3% respectively. At
the second stage, namely a slow-release stage, BMP-2 was released slowly until the end of the observation period. At day 20, the accumulative release amount and release rate of BMP-2 for the 4 groups reached 11.6±0.1, 30.8±0.2, 55.3±0.8, 91.9±1.5 ng and 72.5±2.1, 76.7±1.3, 62.8±1.3, 49.1±1.5% respectively. These results demonstrated that the accumulative release amount of BMP-2 was increased with the increase of BMP-2 concentrations. Although the overall release rate showed a reduction trend, the group of 100 ng/mL showed higher releasing rate than that of 50 ng/mL group after day 10 (day 15 and 20) (Fig. 5B).

In-vitro cell experiment
The SEM images of the MC3T3-E1 cells cultured on various coating surfaces for 1, 4 and 24 h were shown in Figs. 6–8 as a reference index for their adhesion ability in the early stage. After 1 h of culture, MC3T3-E1 cells in 0 ng/mL BMP-2 coating group all presented a round shape (Fig. 6A). In 50 and 100 ng/mL BMP-2 coating groups, cells were also in round shape but began to spread, filopodia and lamellipodia could be observed (Figs. 6B and C). In 200 and 400 ng/mL BMP-2 groups, cells tended to spread into a flat shape. After 4 h of

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Fig. 4 Incorporation efficiencies (IE) of BMP-2 into biomimetic coatings at different concentration of BMP-2.

Fig. 5 Cumulative release profiles of BMP-2 from biomimetic coatings with different concentrations of BMP-2.
A: cumulative release amounts of BMP-2 in 20 days, B: cumulative release rates in 20 days

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Fig. 6 Morphologies of cells cultured on the coatings prepared in SCP containing different concentrations of BMP-2 after 1 h under SEM (1,000×).
A. 0 ng/mL, B. 50 ng/mL, C. 100 ng/mL, D. 200 ng/mL, E. 400 ng/mL.
Fig. 7  Morphologies of cells cultured on the coatings prepared in SCP containing different concentrations of BMP-2 after 4 h under SEM (1,000×).
A. 0 ng/mL, B. 50 ng/mL, C. 100 ng/mL, D. 200 ng/mL, E. 400 ng/mL

Fig. 8  Morphologies of cells cultured on the coatings prepared in SCP containing different concentrations of BMP-2 after 24 h under SEM (1,000×).
A. 0 ng/mL, B. 50 ng/mL, C. 100 ng/mL, D. 200 ng/mL, E. 400 ng/mL

Fig. 9  Cell proliferation state (absorbance at 490 nm wavelength) of MC3T3-E1 cultured on the coatings prepared in SCP containing different concentrations of BMP-2 after 1, 3 and 5 day. *above bars denote statistically difference with the control group (One-way analysis of variance, \( p<0.05 \)), **above bars denote significantly statistically difference with the control group (\( p<0.01 \)).

Fig. 10  ALP activity of MC3T3-E1 cells cultured on the coatings prepared in SCP containing different concentrations of BMP-2 after 7 and 14 day. *above bars denote statistically difference with control group (One-way analysis of variance, \( p<0.05 \)).
culture, cells on all tested surfaces were fully spread out in a polygonal shape. Cells on high BMP-2 concentration coatings tend to present long filopodia (Fig. 7). After 24 h of culture, all cells demonstrated a typical spindle shape of osteoblasts (Fig. 8).

The 490 nm absorbance values (OD_{490}) of the supernatant from each well, which represent the number of MC3T3-E1 cells and reflect cell proliferation status, are plotted in Fig. 9 ((F_d=26.22, p<0.01) (F_a=41.55, p<0.05) (F_d=7.195, p<0.05)). It is showed that the OD_{490} values increased significantly with the time of cell culture. At the same time point, the OD_{490} values showed a dose-dependence on BMP-2 concentrations, increasing with the increase of BMP-2 concentrations.

The ALP activity of MC3T3-E1, an indicator of the cell differentiation ability, is presented in Fig. 10. It can be seen that ALP activities at day 14 were significantly higher than those of day 7. At day 7, no significant difference among the various coating groups was detected while at day 14, the ALP activities of 200 and 400 ng/mL groups were significantly higher than those of the 0, 50 and 100 ng/mL group (F=32.606, p<0.05).

**DISCUSSION**

Due to the verified function of osteogenesis enhancement, BMPs are widely used in orthopedics and dentistry to promote bone defect rehabilitation and osteointegration formation. BMPs are soluble proteins which can be cleared or degraded quickly by body fluid and enzyme. Therefore, their clinical dosage are normally uplifted to a high level (milligram range) far beyond physical range (nanogram range) so as to achieve a satisfied treatment effect, which results in the increase of medical expenses and incidence of unpredicted medical side effects such as neoplasia and ectopic osteogenesis. The use of a delivery system that can retain and sequester BMPs at the target site will hopefully enhance its efficacy and reduce its dosage by localizing the morphogenetic stimulus. In this study, we incorporated BMP-2 into the biomimetic CaP coating on the titanium surface. A sustained release of BMP-2 with enhanced cell proliferation and differentiation was achieved. The biomimetic coating obtained in this study was mainly composed of carbonated CaP, an essential inorganic part of hard tissues with osteo-conductive function. Many studies have demonstrated that apatite coating can facilitate osteogenic differentiation, reduce fibrous encapsulation, and promote bone ingrowth. In addition, as the BMP-2 loading process was conducted in a natural, neutral and physiological environment, the biological activities of BMP-2, i.e. cell adhesion and proliferation, were efficiently preserved. Such biological characteristics of the BMP-2 incorporated CaP coating offer an excellent biocompatibility for titanium based materials.

A sustained release of BMP-2 over 20 days by changing the amount of BMP-2 in the mineralization solution was achieved in this study. The release behavior of the bioactive molecules is affected by the incorporation efficacy, degradation speed of the coating, as well as the site where the bioactive molecules are bound or absorbed. Generally, there are three release mechanisms: 1) nature diffusion resulted by the concentration gradient; 2) release along the dissolving of the coating; and 3) release along the degradation of the coating caused by various enzymes, osteoclast or lymphocyte. In our study, the releasing profiles of all the groups consist of two stages: the initial burst release stage and the latter slow release stage. The burst release in the first three days was resulted from the diffusion of BMP-2 molecules which were physical adsorbed on the surface of the apatite coatings. After three days burst release, the release rate was significantly reduced and then kept in a slow, stable level, lasting for over 20 days, forming a slow release stage. The release of BMP-2 in this stage is thought to occur with the dissolving or degradation of CaP. Such release pattern is consistent with the previous study in which 200 ng/mL of BMP-2 was incorporated into the polycaprolactone-collagen scaffold. In addition, compared to the over 80% burst release reported before in non-control-release system such as absorbed collagen scaffolds, the accumulated burst release rate of BMP-2 in the first 3 day decreased down to 30–60% for the various concentration groups. This proves the effectiveness of this biomimetic CaP coating in fixing BMP-2 and controlling the release of BMP-2. More importantly, the CaP coating can be naturally degraded in the physical environment, and the BMP-2 incorporated or locked inside the coating could release in a sustained manner over time with the degradation of apatite coating. In this way, minimal diffusion of BMP-2 from body liquid could be ensured, and the local concentration could be maintained in a certain level to ensure its physiological effect.

Biological activity is a crucial point for the application of surface-modified titanium in orthopedics and dentistry. Our results showed that the BMP-2 incorporated CaP coating could enhance osteogenesis. The incorporation of BMP-2 inside the CaP coating facilitated osteoblast spreading. The proliferation and differentiation capability of the osteoblastic adhered on the HA/BMP-2 coated Ti surfaces showed a BMP-2 concentration-dependent manner in the late stages, i.e. at day 3 and 5 of cell proliferation test and at day 14 of ALP test. The CCK-8 results confirmed that within the BMP-2 dose range we used in this experiment, the cumulative BMP-2 at the initial fast release stage could promote cell proliferation, maintaining in an effective and safe level. From the 14 day ALP assay results, it can be seen that although the amount of BMP-2 released is relatively low in the second stage, it is sufficient to promote osteoblasts differentiation (Fig. 10). In spite that the cell culture only covers 14 days, the growth and differential potential of cells have already been activated by the early initial stimulation, and this activity will maintain for a long time even if the later BMP-2 concentration is in a relatively low level. Therefore, it can still be predicted that this favorable cell growth trend can be maintained during the whole process of BMP-2 release.
This result has also been proved by Lee et al.\textsuperscript{20}. After 14 days/cell culture, significant higher ALP activity was found in 200 and 400 ng/mL BMP-2 group. For the rest groups, no significant difference could be observed in the early stages among 0, 50 ng/mL and 100 ng/mL BMP-2 groups, this might because that the content of BMP-2 was too low to take effect. In spite of this, it still could be concluded that with the aid of an effective carrier, \textit{i.e.}, hydroxyapatite, BMP-2 could work in a relatively low, nanogram scale dose range.

In summary, by biomimetic co-deposition technique, a CaP coating composed of hydroxyapatite could be formed on titanium surface, low-dose of BMP-2 could be successfully incorporated into the coating during the co-deposition process without losing its bioactivity. By the aid of this osteoconductive and biodegradable CaP coating, BMP-2 was released in a sustained manner. The hybrid coating of BMP-2/hydroxyapatite significantly promoted the proliferation and differentiation ability of MC3T3-E1 on the titanium surface. Such BMP-2 loaded apatite coating has the potential to be applied in dental and orthopedic implantation.

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